ASSESSING THE CONFORMATIONAL SHIFT OF TRANSFER RNA- PHENYLALANINE IN THE PRESENCE OF IRON(II) AND MAGNESIUM(II)

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LIST OF SYMBOLS AND ABBREVIATIONS

SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
CD	circular dichroism
$tRNA^{Phe}$	transfer RNA – phenylalanine
aPTC	ancient peptidyl transferase center

SUMMARY

Association of the small subunit and large subunit of the ribosome activates the synthesis and extension of a polypeptide through peptide bond formationat the peptidyl transferase center (PTC). This complex incorporates transfer RNA (tRNA) for the transfer of an amino acid to a growing polypeptide chain. Structure-based comparison of the large subunits (LSUs) of prokaryotic and archaeal species reveals a structurally conserved, magnesium rich core near the site of peptidyl transfer. The structural change of tRNA in the presence of Mg^{2+} could have serious implications for its interaction with the PTC for peptidyl transfer. The binding and conformational shift of tRNA with Mg^{2+} was observed through the use of circular dichrosim spectroscopy. tRNA was first folded in the presence of low concentration of sodium and then titrated with increasing concentrations of Mg^{2+} as circuilar dichroism spectra were measured. A distinct conformational shift was observed between 12 and 17.5μM of Mg^{2+}, indicating the tight binding of a transition type II metal. An SDS-PAGE gel confirmed the change in molar ellipticity observed was not due to degradation of tRNA.

CHAPTER 1

INTRODUCTION

Abiogenesis

The direction that we take with our future can hinge on our knowledge of where we came from and how we began as a species. Some scientists believe that the beginning of complex protein-based life is bridged from the RNA-based world through the formation of the ribosome, the site of translation and protein synthesis. Through the understanding of the early mechanisms of translation , we can better understand both the origin of life on our planet and possible means through which life could evolve in different conditions throughout the universe. Though the ribosome has developed to accommodate the translational requirement of different groups of organisms through billions of years of evolution. Basic motifs common to all species are key in determining the makeup of a possible universal ribosomal ancestor. The ribosome in prokaryotes and eukaryotes is comprised of a large ribosomal subunit and small subunit which complex in the presence of a messenger RNA template to initiate protein translation. Although the sizes of these subunits differ between eukaryotes and prokaryotes (50S and 30S in prokaryotes), they complex and form the site of aminoacyl transfer in similar ways.

Transfer RNA and the Ribosome

Basic models of what the early ribosome was comprised of and how it facilitated protein synthesis have been constructed through the analysis of ribosomal crystal structures of bacterial and archaeal species (Bokov & Steinberg, 2009). These analyses have led to the

identification of key sequences common to all species and integral to the formation of an ancient peptidyl transferase center, termed the aPTC (Bowman, Lenz, et al. 2012). The conformations of ribosomal RNA and transfer RNA in three-dimensional space are critical to the understanding of their mechanisms of interaction.

tRNA Conformations

Metal ions such as sodium and magnesium interact with the phosphate backbone of RNA to give rise to different conformations (Romer and Hatch, 1975). Monovalent cations, such as Na^+, can stabilize the cloverleaf structure of tRNA (Figure 1). The cloverleaf secondary structure of tRNA in the presence of monovalent ions contains three loops of single stranded RNA called the D, T, and anticodon loops (Sherlin, Bullock, et al., 2000). It also contains the acceptor stem, which binds a specified amino acid by the interaction of aminoacyl-tRNA synthetase with the 3'-OH (Sherlin, Bullock, et al., 2000).

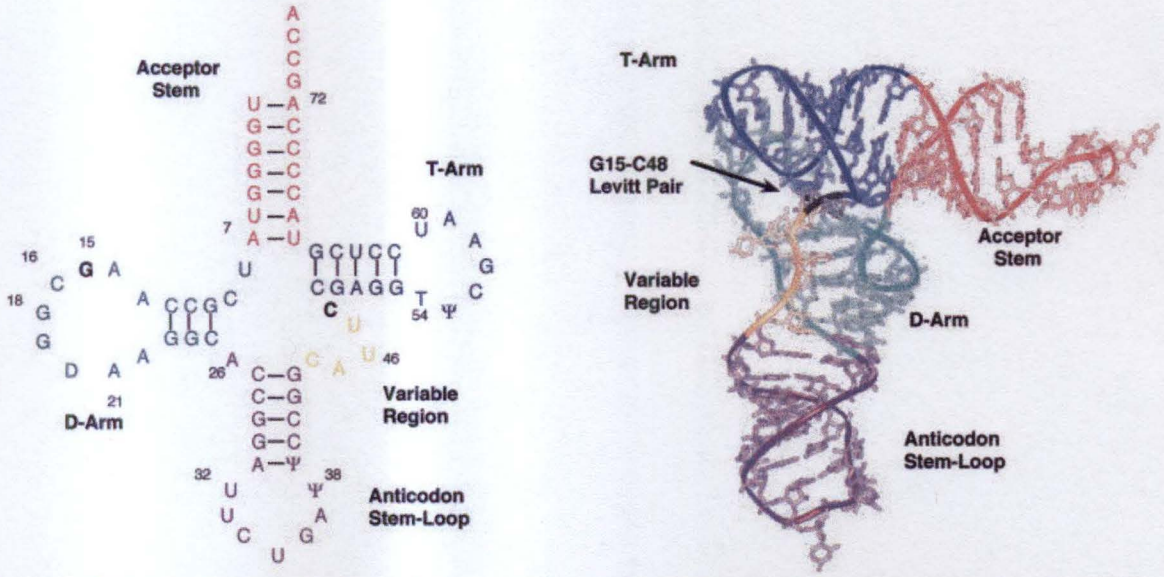

Figure 1. Cloverleaf Structure. Secondary cloverleaf structure (left) and tertiary cloverleaf structure (right) of tRNA.

The L-shaped tertiary structure of tRNA allows for binding to the peptidyl and aminoacyl sites of the peptidyl transferase center for protein translation (Xiao, Murakimi, et al., 2008). The presence and binding of Mg^{2+} allows for the folding of tRNA to its more functionally relevant L-shaped tertiary structure. Divalent cations that were prevalent in the Precambrian period and that likely interacted with early biomolecules were iron and magnesium (Shaw, et al., 1986). The study of how iron and magnesium bind and interact with tRNA is critical to understanding the beginning of cellular life. The aim of this project is to assess how the presence of Mg^{2+} affects the conformation of $tRNA^{Phe}$ (yeast).

CHAPTER 2

BINDING OF DIVALENT CATIONS

Mg^{2+} Binding

The binding of magnesium(II) to $tRNA^{Phe}$ and other RNA molecules has been studied considerably since the 1970s and the existence of multiple types of binding has been identified. Both tight binding at concentrations as low as 4μM and loose binding at high concentrations of 20mM have been shown to cause four distinct transitions in the structure of $tRNA^{Phe}$ (Serebrov et al., 2001). Based on the crystal structure (Figure 2), two binding pockets of high electrostatic potential are likely located between the D- and T-loops of tRNA and between the 5' acceptor strand and the D-stem (Jack et al. 1977).

Conformational Shift in Circular Dichroism

Transfer RNA^{Phe} has been shown to demonstrate a distinct conformational change in the presence of Mg^{2+} at 264 nm in circular dichroism studies, so an appropriate range to observe this signal was decided to be 220 nm to 340nm due to the high level of noise present below 220 nm (Serebrov et al., 2001). The shift observed at this wavelength is not only characterized by a increase in peak height at 264nm with increasing magnesium concentration, but also an increase in peak height just above 260nm (Sosnick, Fang, Shelton, 2000). This leads to the conclusion that a distinct conformation of tRNA is formed in the presence of Mg^{2+} (Sosnick, Fang, Shelton, 2000).

The Effect of Temperature

Temperature was also determined to be a major factor in the degree of the conformational shift in the presence of Mg^{2+}. Experiments performed at 15 °C served as a standard, but those performed at 40 °C observed a much more dramatic peak height and wavelength shift (Romer and Hach, 1975). Although this increase in temperature improves the characterization of the shift in the presence of Mg^{2+}, high temperatures can lead to the degradation of tRNA in the sample.

Previous Experiments

My own research lab has performed similar experiments to test the conformational shift of ribosomal RNA sequences in the presence of Mg^{2+}. They have shown that RNA samples with concentrations corresponding to approximately 1.0 OD at 260nm in a UV-Vis Spectrum provide the most accurate signal to noise ratio for CD characterization of an RNA conformational shift (Bowman, Lenz et al., 2012). A concentration of approximately 5.1μM $tRNA^{Phe}$ was determined to give an OD of approximately 1.0 unit at 260nm. At approximately 21.5μM magnesium(II) seems to cause a conformational change in tRNA (Maglott, Sanmitra, Deo et al., 1998) (Sampson and Uhlenbeck, 1988). This helped in the determination of the concentrations of Mg^{2+} to be tested in the experiment (2μM - 200μM). $tRNA^{Phe}$ was folded in the presence of monovalent cations prior to incubation with magnesium(II) to better observe the desired conformational shift to a cloverleaf structure (Quigley, Teeter, Rich, 1978). The sodium buffers utilized in this experiment served as a model for this experiment.

CHAPTER 3

MATERIALS AND METHODS

The observation of conformational change in tRNA-Phe in the presence of magnesium(II) will be measured through circular dichroism methods. Transfer RNA-Phe, obtained from Sigma Aldrich and purified through the Zymo RNA purification system, will be diluted down to a concentration of approximately 2.6μM for optimum signal-to-noise ratio. This will be dissolved in a buffer of sodium cacodylate (10mM, pH 6.0) and sodium chloride (22mM) to obtain a stable conformation of tRNA in the presence of a monovalent cation (Na+). Folding of the tRNA from this conformation will be induced through heating in a heating block initially at 70°C for 5 minutes with a chelating resin and gradual cooling over one hour. The sample will be centrifuged to remove divalent cations present before the experiment through binding to the resin. tRNA will then be incubated in a 1cm path length CD cuvette with stock solutions containing magnesium chloride of concentrations: 0, 2μM, 4μM, 6μM, 8μM, 10μM, 12μM, 17.5μM, 25μM, 50μM, 75μM, 100μM, 150μM, and 200μM.

CD spectral data from 220nm to 340nm will then be obtained for the samples at 15°C with a scanning speed of 500nm/min and averaged over five accumulated spectra in a Jasco Circular Dichroism Spectrometer. This same experiment will be repeated with magnesium chloride to compare the difference in conformational change and CD signal. The peak height and shift give a clear indication of three dimension conformational change in transfer RNA. After CD experiments, samples will be mixed and centrifuged again with a

metal ion chelating resin to remove divalent ions. These samples can then be checked for degradation due to metal ions by SDS-PAGE.

CHAPTER 4

RESULTS

Circular Dichrosim Spectral Measurements

Conformational change of $tRNA^{Phe}$ in the presence of Mg^{2+} was measured as a function of $[Mg^{2+}]$. Molar ellipticity of tRNA in presence and absence of Mg^{2+} was measured in the wavelength range of 220 to 340nm. The change in the CD spectrum was measured as a function of Mg^{2+} concentration ranging from 0μM to 300μM. The majority of CD spectra fall within two clusters of overlapping spectra in the range of 260 to 280nm (Figure 3). A principle maximum and minimum occurs in all samples at wavelengths of 278 and 235nm, respectively (Figure 3). The transition between these two clusters occurs between Mg^{2+} concentrations of 12 and 17.5μM (Figure 3).

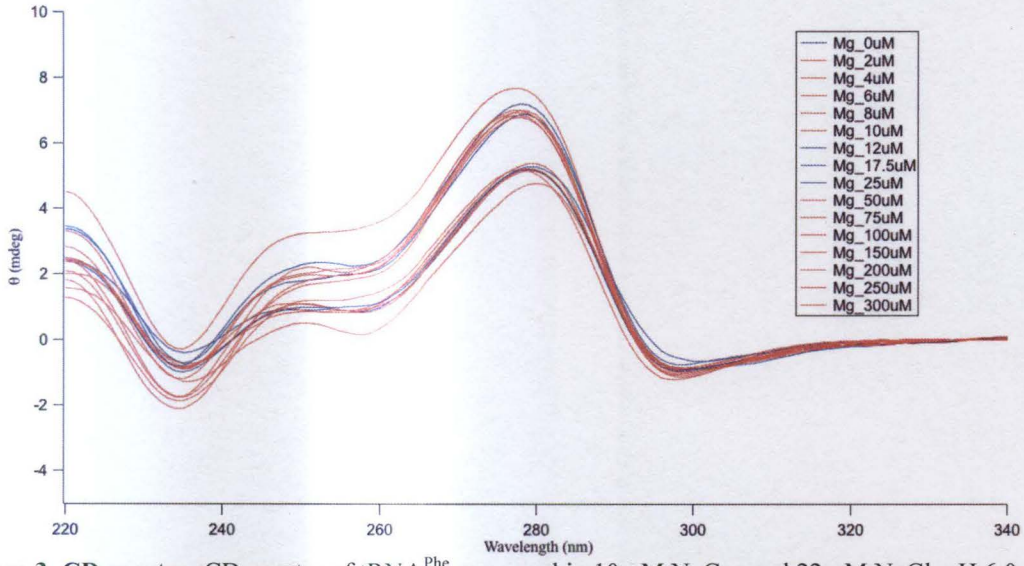

Figure 3. CD spectra. CD spectra of $tRNA^{Phe}$ measured in 10mM NaCac and 22mM NaCl, pH 6.0, as a function of $[Mg^{2+}]$ at 15°C. Overlapping of spectra occurs mostly between 260 and 280nm, where two clusters of spectra formed. Spectra used to determine the location of the transition are in blue : 0, 12, 17.5, 25μM. Blue spectra represent four concentrations surrounding the sigmoidal transition in structure change.

A closer view of the maxima in the range of 260 to 300nm shows the transition between the two CD spectra clusters (Figure 4). Concentrations of 0 and 12μM of Mg^{2+} overlap greatly in the region of 260 to 280nm, showing that no significant molar ellipticity change occurs below 12μM in this range (Figure 4).

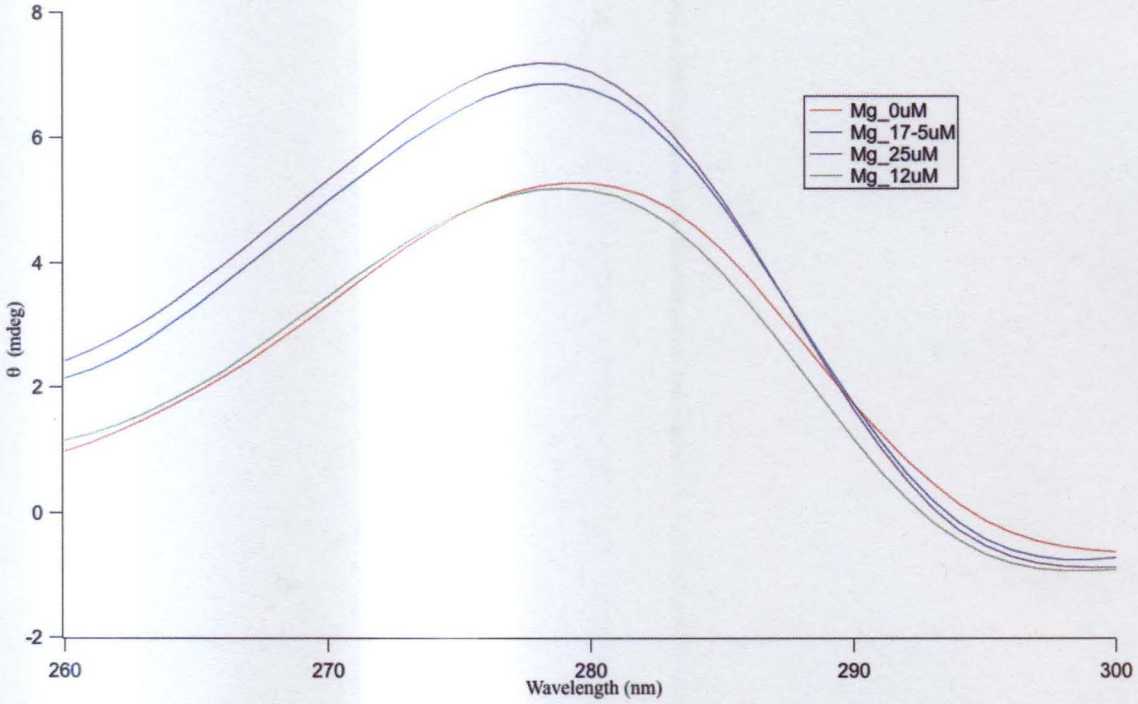

Figure 4. Molar Ellipticity Shift. Zoomed in perspective of the CD spectra for 0, 12, 17.5, and 25μM of Mg^{2+} in 32mM Na^{+} at pH 6.0 and 15°C. The data display the two clusters formed by the full data set and the transition in molar ellipticity above 12μM.

Sigmoidal Transition of Molar Ellipticity

In comparing the molar ellipticity of $tRNA^{Phe}$ at 275nm with the concentration of magnesium(II), a roughly sigmoidal transition in molar ellipticity is observed. Low concentrations of Mg^{2+} up to 12μM form a baseline of molar ellipticity at 4.7mdeg (Figure 5). A sigmoidal transition is observed between 12 and 17.5μM, indicative of the formation of the cloverleaf structure of tRNA (Figure 5).

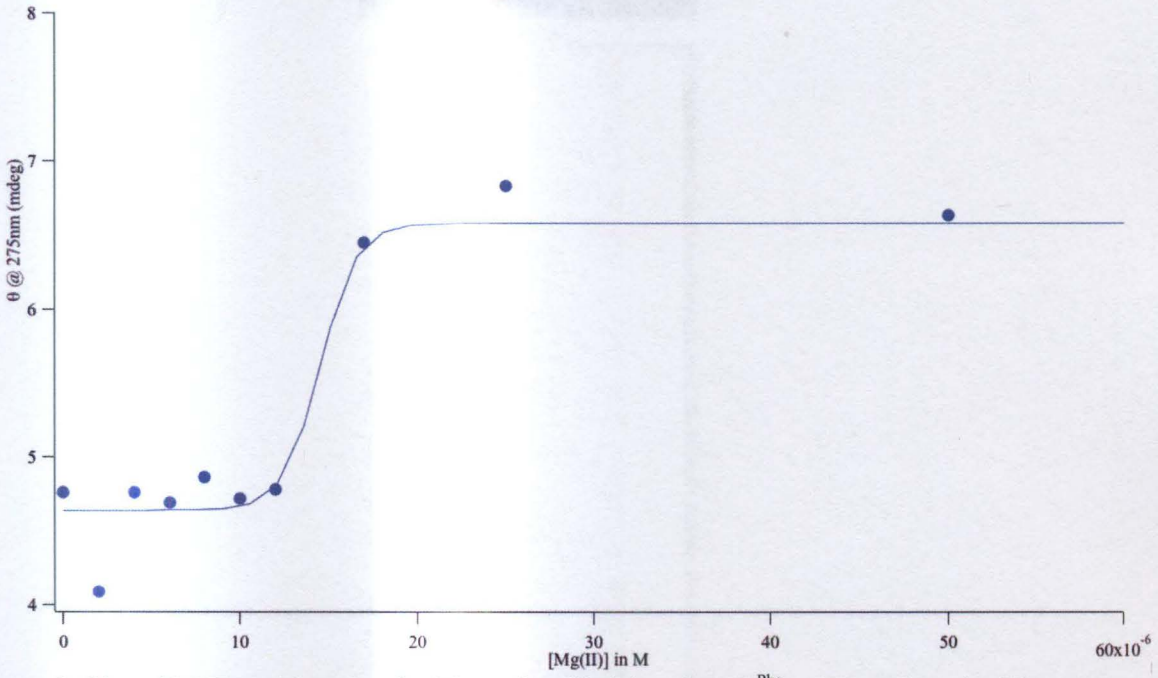

Figure 5. Sigmoidal Transition. Graph of the molar ellipticity of $tRNA^{Phe}$ at 275nm from the CD spectra as a function of $[Mg^{2+}]$. The molar ellipticities at concentrations between 0 and 12μM form a baseline of similar molar ellipticity. A strong increase in molar ellipticity is observed at 17.5μM, indicating that a sigmoidal transition occurs between 12 and 17.5μM.

Urea-PAGE

To examine the degradation of tRNA in presence of Mg(II), an 8% urea-PAGE gel was run. Compared to a high-range single stranded RNA ladder, $tRNA^{Phe}$ with concentrations ranging from 0 to 2.5mM of Mg^{2+} showed no significant difference in the size of major bands.

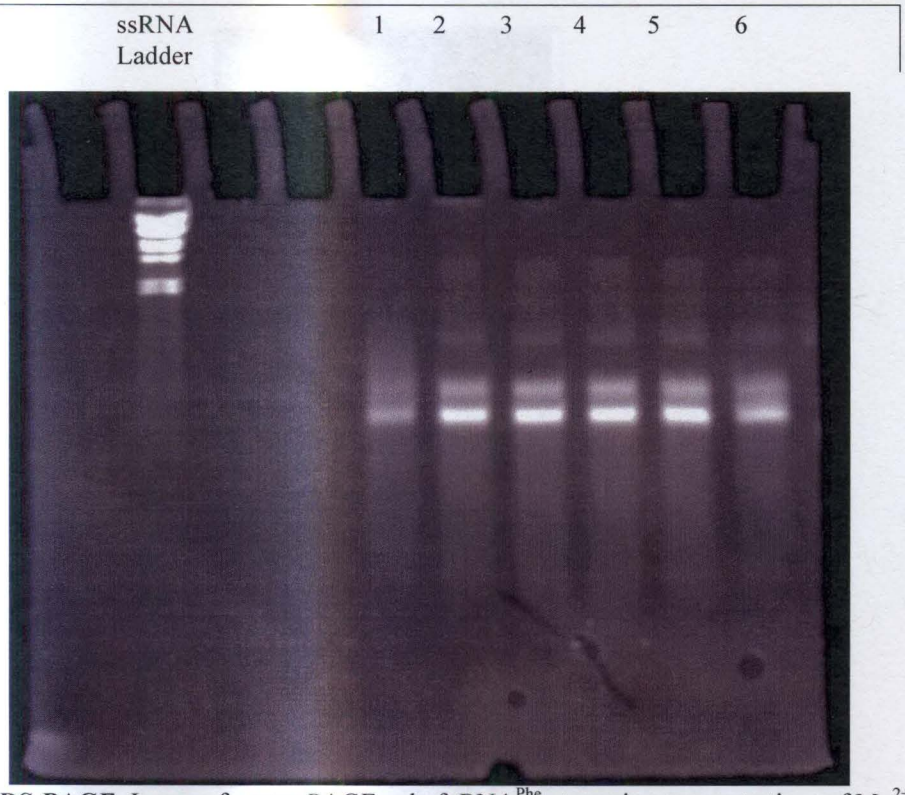

Figure 6. SDS-PAGE. Image of a urea-PAGE gel of $tRNA^{Phe}$ at varying concentrations of Mg^{2+} after incubation and the completion of the CD experiment. A high range single stranded RNA ladder was compared to lanes 1-6, which corresponded to $tRNA^{Phe}$ incubated with Mg^{2+} concentrations of 2.5, 1.25, 0.625, 0.313, 0.156, and 0mM respectively.

CHAPTER 5

DISCUSSION

Binding of Mg^{2+} in tRNA Transitions

Tight binding of Mg^{2+} occurs at tight pockets of negative charge density of tRNA, where they are retained by only Columbic interactions according to an electrostatic model (Serebrov et al., 2001). Based on this model, Mg^{2+} competes with monovalent cations present for binding sites. Mg^{2+} displays cooperative binding to tRNA through increasing the compactness and charge density of tRNA upon binding (Serebrov et al., 2001). Four different types of binding sites for Mg^{2+} on tRNA have been identified, two that display tight binding at 4μM and 16μM, and two that display loose binding at 2mM and 20mM (Serebrov et al., 2001). The large transition in molar ellipticity between Mg^{2+} concentrations of 12 and 17.5μM is indicative of a type II transition, which was caused by tight binding of Mg^{2+} in previous experiments (Serebrov et al., 2001).

Metal Ions and tRNA Structure

The tertiary cloverleaf structure of tRNAs remain highly similar regardless of posttranscriptional modifications due to the high conservation of single-stranded loop residues of the three loops. However, the stability of these tRNAs can vary significantly depending on the size and charge of the amino acid residue added. The use of the electron releasing phenylalanine group gives a better picture of the structure of most tRNAs with uncharged groups. Although Na^{+} buffer at low concentration forms the stabilized cloverleaf structure, it does not cause a conformational shift to the native tRNA structure that has been

previously observed through the addition of Mg^{2+}. Mg^{2+} has been shown to be critical to the formation of native L structure of tRNA through tight binding.

Verification of Results

The shift in molar ellipticity with increasing Mg^{2+} concentration was shown to not result in RNA degradation through a urea-PAGE gel of tRNA incubated with varying concentrations of Mg^{2+}. Degradation of the tRNA by the presence of Mg^{2+} can lead to quenching effects, which could cause an increase in the molar ellipticity due to breakup of the tRNA. However, the band sizes of samples with Mg^{2+} concentrations up to 2.5mM showed no signs of degradation with similar band sizes conserved among all samples.

CHAPTER 6

FUTURE WORK

Since Fe^{2+} constituted a major part of the Earth's crust before the Great Oxidative Event, its study would be invaluable to evaluating the interactions of tRNA in early protein synthesis machinery. The binding of $tRNA^{Phe}$ and other RNA molecules to Mg^{2+} has been studied considerably since the 1970s, yet relatively little study has been done on the conformational changes of these biomolecules in the presence of Fe^{2+}. This can be attributed to the ease with which iron(II) is oxidized to iron(III) in exposure to atmospheric oxygen. The creation of an oxygen free environment for Fe^{2+} to bind tRNA is critical for the success of this experiment because the presence of Fe^{3+} can lead to severe degradation of $tRNA^{Phe}$. To maintain an anoxic environment, the sample will be sealed and bubbled with argon to remove atmospheric oxygen in the absence of Fe^{2+} in the CD cuvette itself. Subsequent titration of this single sample with increasing concentrations of Fe^{2+} will be performed with stock solutions of Fe^{2+}, which have also been bubbled with argon prior to the experiment. Melts will also be performed to determine the temperature dependence of Mg^{2+} and Fe^{2+} binding to tRNA. Previous experiments have shown that Mg^{2+} binding causes a more dramatic shift in molar ellipticity in CD measurements at 40°C compared to 15°C, indicating the need for a high range of temperatures tested.

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